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(54) CELL DIVISION INHIBITORS AND PROCESS FOR PRODUCING THE SAME

(57) The present invention relates to a cell division inhibitor comprising various dehydrodiketopiperazines such as dehydrophenylahistin, or analogs thereof as an active ingredient, and a dehydrogenase and a method for producing the same inhibitor.

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Description

Technical Field

[0001] The present invention relates to a cell division inhibitor (a cell cycle inhibitor) and an antitumor agent, and a method of producing them using enzymes.

Background

[0002] The growth and differentiation of cells constituting a human body are strictly controlled in order to maintain homeostasis. Cells divide or proliferate by repeating a cell cycle consisting of a certain process comprising M period, G1 period, S period and G2 period. A defect in the control mechanism of this cell cycle results in the development of cancer or immune disease.

[0003] Lately, the control mechanism of cell cycle is clarifying at a molecular level, and it is known that a substance controlling a cell cycle possibly can be used as an antitumor agent or an immunosuppressive agent. In recent years, as an antitumor agent or a lead compound thereof, the spotlight has centered on a substance, such as pacritaxel, vincristine or vinblastine, inhibiting the function of tubulin which is one of cytoskeleton proteins playing a major role in precisely distributing a replicated gene into a daughter cell in a cell division stage.

[0004] Fukushima *et al.* have found that albonoursin has an antitumor activity and an antibacterial activity (Fukushima *et al., J. Antibiotics*, Vol.26, pp.175, 1973), while Kobayashi *et al.* have found that albonoursin acts to inhibit the pronuclear fusion between a female nucleus and a male nucleus (Kobayashi *et al., A Summary of the Symposium on the Chemistry of Natural Products*, P51, 1989). Furthermore, Kanzaki *et al.* have found that tetradehydrocyclo (Phe-Phe) exhibits sea urchin embryo division inhibitory activity (*A Summary of the Symposium of the Society for Actinomycetes Japan*, P42, 1999).

[0005] Kanoh *et al.* have found that, filamentous bacteria, *Aspergillus ustus* NSC-F037 and *Aspergillus ustus* NSC-F038, which were isolated from the soil in Kanagawa prefecture, produce a novel antitumor substance phenylahistin, and have determined the structure of this substance. Phenylahistin molecules have a chiral carbon atom, and as a result of a thorough examination, Kanoh *et al.* have further discovered that phenylahistin produced by the above bacteria is a mixture of (-)- phenylahistin and (+)-phenylahistin, and that the antitumor acitivity of (-)- phenylahistin is approx. 30-100 times stronger than that of (+)- phenylahistin (Japanese Patent Application Laying-Open (kokai) No. 10-130266, Kanoh *et al., Bioorganic & Medicinal Chemistry Letters*, Vol.7, No.22, pp.2847-2852, 1997 ; Kanoh *et al., Bioscience Biotechnology Biochemistry*, Vol.63, No. 6, pp.1130-1133, 1999). Further, they have found that (-)- phenylahistin inhibits the polymerization of tubulin (Kanoh *et al., The Journal of Antibiotics*, Vol. 52, No. 2, pp.134-141, 1999). Furthermore, Kanoh et al. have examined the antitumor effect of (-)- phenylahistin, using a cancer cell transplanted model animal, and have shown that (-)- phenylahistin has a certain degree of antitumor activity (Kanoh *et al., Bioscience Biotechnology Biochemistry*, Vol.63, No. 6, pp.1130-1133, 1999). From a clinical position, however, an agent having a stronger antitumor activity than (-)-phenylahistin is desirable.

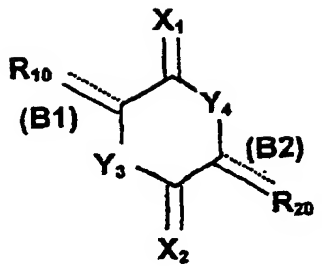
Disclosure of the Invention

[0006] The object of the present invention is to provide a cell division inhibitor having a stronger cell cycle inhibitory activity, particularly antitumor activity, and a method of producing the inhibitor using enzymes.

[0007] As a result of thorough analysis by the present inventors to achieve the above object, it has been found that various dehydrodiketopiperazines such as dehydrophenylahistin or affinities thereof have a stronger cell cycle inhibitory activity than (-)- phenylahistin, and have completed the present invention.

[0008] That is to say, the present invention comprises each of the following inventions.

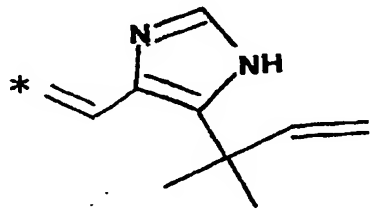
(1) A cell division inhibitor comprising, as an active ingredient, a compound of formula (I) or pharmaceutically acceptable salt thereof:



(I)

wherein

each of X_1 and X_2 is independently oxygen or sulfur;
Y_3 is oxygen, sulfur, $-NR_3-$ or $-CR_{31}R_{32}-$;
Y_4 is oxygen, sulfur, $-NR_4-$ or $-CR_{41}R_{42}-$;
R_{10} is halogen, C_{1-25} alkyl, C_{2-25} alkenyl, C_{2-25} alkynyl, C_{1-25} alkoxy, aralkyl, hydroxyl, amino, nitro or aryl, which may be substituted with other substituent(s), and a part of the carbon chain of R_{10} may be branched or cyclized, or may comprise a heteroatom;
R_{20} is halogen, C_{1-25} alkyl, C_{2-25} alkenyl, C_{2-25} alkynyl, C_{1-25} alkoxy, aralkyl, hydroxyl, amino, nitro or aryl, which may be substituted with other substituent(s), and a part of the carbon chain of R_{20} may be branched or cyclized, or may comprise a heteroatom;
each of R_3 and R_4 is independently hydrogen, halogen, C_{1-25} alkyl, C_{2-25} alkenyl, C_{2-25} alkynyl, C_{1-25} alkoxy, aralkyl, hydroxyl, amino, nitro or aryl, which may be substituted with other substituent(s), and a part of the carbon chain may be branched or cyclized, or may comprise a heteroatom;
each of R_{31}, R_{32}, R_{41} and R_{42} is independently hydrogen, halogen, C_{1-25} alkyl, C_{2-25} alkenyl, C_{2-25} alkynyl, C_{1-25} alkoxy, aralkyl, hydroxyl, amino, nitro or aryl, which may be substituted with other substituent(s), and a part of the carbon chain may be branched or cyclized, or may comprise a heteroatom;
R_{10} and any of R_3, R_{31} and R_{32} may form a ring;
R_{20} and any of R_4, R_{41} and R_{42} may form a ring;
each of (B1) and (B2) independently represents a carbon-carbon single bond or a carbon-carbon double bond, wherein at least one represents a carbon-carbon double bond with E or Z configuration;
at least one of the above groups may have a protecting group capable of decomposing *in vivo*, except in the case where each of X_1 and X_2 is oxygen, each of Y_3 and Y_4 is -NH-, R_{10} is benzyl, each of (B1) and (B2) is a carbon-carbon double bond, and R_{20} is isobutyl or benzyl, and in the case where each of X_1 and X_2 is oxygen, each of Y_3 and Y_4 is -NH-, R_{10} is benzyl, (B1) is a carbon-carbon single bond, (B2) is a carbon-carbon Z double bond, and R_{20} is a group shown in the following formula (a):



(a)

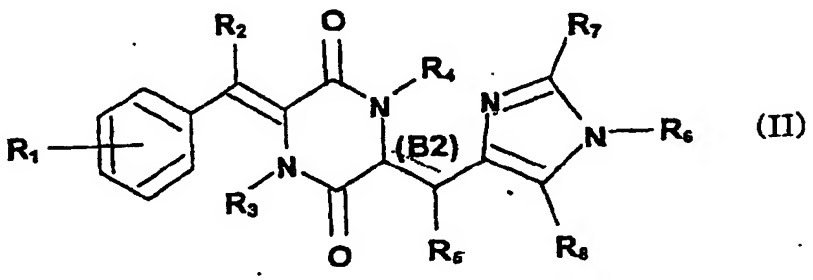
wherein * represents a bonding position.

(2) The cell division inhibitor according to item 1 above wherein, in the formula (I), each of (B1) and (B2) is a carbon-carbon double bond.

(3) The cell division inhibitor according to item 1 or 2 above wherein, in the formula (I), each of X_1 and X_2 is oxygen, Y_3 is $-NR_3-$, and Y_4 is $-NR_4-$.

(4) The cell division inhibitor according to item 3 above wherein, in the formula (I), each of Y_3 and Y_4 is -NH-.

(5) A cell division inhibitor comprising, as an active ingredient, a compound of formula (II) or an E form thereof, or pharmaceutically acceptable salt thereof:



(II)

wherein

R_1 is hydrogen, halogen, C_{1-25} alkyl, C_{2-25} alkenyl, C_{2-25} alkynyl, C_{1-25} alkoxy, aralkyl, hydroxyl, amino, nitro or aryl, which may be substituted with other substituent(s), and a part of the carbon chain of R_1 may be branched or cyclized, or may comprise a heteroatom, and further R_1 may be one atom or group, or at most 5 identical or different atoms or groups, and the atoms or groups may mutually form a ring;
R_2 is hydrogen, halogen, C_{1-25} alkyl, C_{2-25} alkenyl, C_{2-25} alkynyl, C_{1-25} alkoxy, aralkyl, hydroxyl, amino, nitro or aryl, which may be substituted with other substituent(s), and a part of the carbon chain of R_2 may be branched or cyclized, or may comprise a heteroatom;
each of R_3 and R_4 is independently hydrogen, halogen, C_{1-25} alkyl, C_{2-25} alkenyl, C_{2-25} alkynyl, C_{1-25} alkoxy, aralkyl, hydroxyl, amino, nitro or aryl, which may be substituted with other substituent(s), and a part of the carbon chain may be branched or cyclized, or may comprise a heteroatom;
R_5 is hydrogen, halogen, C_{1-25} alkyl, C_{2-25} alkenyl, C_{2-25} alkynyl, C_{1-25} alkoxy, aralkyl, hydroxyl, amino, nitro or aryl, which may be substituted with other substituent(s), and a part of the carbon chain of R_5 may be branched or cyclized, or may comprise a heteroatom;
R_6 is hydrogen, halogen, C_{1-25} alkyl, C_{2-25} alkenyl, C_{2-25} alkynyl, C_{1-25} alkoxy, aralkyl, hydroxyl, amino, nitro or aryl, which may be substituted with other substituent(s), and a part of the carbon chain of R_6 may be branched or cyclized, or may comprise a heteroatom;
each of R_7 and R_8 is independently hydrogen, halogen, C_{1-25} alkyl, C_{2-25} alkenyl, C_{2-25} alkynyl, C_{1-25} alkoxy, aralkyl, hydroxyl, amino, nitro or aryl, which may be substituted with other substituent(s), and a part of the carbon chain may be branched or cyclized, or may comprise a heteroatom;
R_2 and R_3 may form a ring;
R_4 and any of R_5, R_6, R_7 and R_8 may form a ring;
(B2) represents a carbon-carbon single bond or a carbon-carbon double bond;
at least one of the above groups may have a protecting group capable of decomposing *in vivo*.

(6) The cell division inhibitor according to item 5 above wherein, in the formula (II), (B2) is a carbon-carbon double bond.

(7) The cell division inhibitor according to item 6 above wherein, in the formula (II), at least one of R_7 and R_8 is 1, 1-dimethyl-2-propenyl.

(8) The cell division inhibitor according to any one of items 1-7 above wherein it is an antitumor agent.

(9) A dehydrogenase which has an activity to convert a compound represented by the above formula (I) wherein at least one of (B1) and (B2) is a carbon-carbon single bond, or by the above formula (II) wherein (B2) is a carbon-carbon single bond into a compound wherein the carbon-carbon single bond(s) is replaced with a carbon-carbon double bond(s).

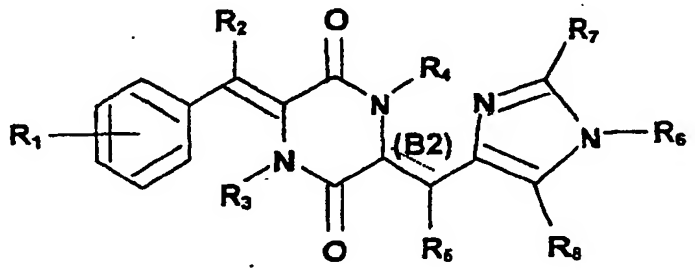
(10)The dehydrogenase according to item 9 above whose molecular weight is 700-800 kDa.

(11)The dehydrogenase according to item 9 or 10 above which is produced by *Streptomyces albulus*.

(12)A method of producing the cell division inhibitor according to any one of items 1-8 above, which comprises using, as a substrate, a compound represented by the above formula (I) wherein at least one of (B1) and (B2) is a carbon-carbon single bond, or a compound represented by the above formula (II) wherein (B2) is a carbon-carbon single bond, and converting the carbon-carbon single bond to a carbon-carbon double bond by use of a cell, cell-free extract or enzyme solution containing the dehydrogenase according to any one of items 9-11 above.

(13)The method according to item 12 above wherein the dehydrogenase of item 11 above is used.

(14)A compound of formula (II) or an E form thereof, or pharmaceutically acceptable salt thereof:



(II)

wherein

R_1 is hydrogen, halogen, C_{1-25} alkyl, C_{2-25} alkenyl, C_{2-25} alkynyl, C_{1-25} alkoxy, aralkyl, hydroxyl, amino, nitro or aryl, which may be substituted with other substituent(s), and a part of the carbon chain of R_1 may be branched or cyclized, or may comprise a heteroatom, and further R_1 may be one atom or group, or at most 5 identical or different atoms or groups, and the atoms or groups may mutually form a ring;
R_2 is hydrogen, halogen, C_{1-25} alkyl, C_{2-25} alkenyl, C_{2-25} alkynyl, C_{1-25} alkoxy, aralkyl, hydroxyl, amino, nitro or aryl, which may be substituted with other substituent(s), and a part of the carbon chain of R_2 may be branched or cyclized, or may comprise a heteroatom;
each of R_3 and R_4 is independently hydrogen, halogen, C_{1-25} alkyl, C_{2-25} alkenyl, C_{2-25} alkynyl, C_{1-25} alkoxy, aralkyl, hydroxyl, amino, nitro or aryl, which may be substituted with other substituent(s), and a part of the carbon chain may be branched or cyclized, or may comprise a heteroatom;
R_5 is hydrogen, halogen, C_{1-25} alkyl, C_{2-25} alkenyl, C_{2-25} alkynyl, C_{1-25} alkoxy, aralkyl, hydroxyl, amino, nitro or aryl, which may be substituted with other substituent(s), and a part of the carbon chain of R_5 may be branched or cyclized, or may comprise a heteroatom;
R_6 is hydrogen, halogen, C_{1-25} alkyl, C_{2-25} alkenyl, C_{2-25} alkynyl, C_{1-25} alkoxy, aralkyl, hydroxyl, amino, nitro or aryl, which may be substituted with other substituent(s), and a part of the carbon chain of R_6 may be branched or cyclized, or may comprise a heteroatom;
each of R_7 and R_8 is independently hydrogen, halogen, C_{1-25} alkyl, C_{2-25} alkenyl, C_{2-25} alkynyl, C_{1-25} alkoxy, aralkyl, hydroxyl, amino, nitro or aryl, which may be substituted with other substituent(s), and a part of the carbon chain may be branched or cyclized, or may comprise a heteroatom;
R_2 and R_3 may form a ring;
R_4 and any of R_5, R_6, R_7 and R_8 may form a ring;
(B2) represents a carbon-carbon single bond or a carbon-carbon double bond;

at least one of the above groups may have a protecting group capable of decomposing *in vivo*.

[0009] The details of the present invention are disclosed below.

[0010] First of all, regarding various definitions which the present invention comprises, appropriate examples and explanations are provided below.

[0011] The term "halogen" appearing in the formulas (I) and (II) means fluorine, chlorine, bromine or iodine, unless otherwise specified.

[0012] C_{1-25} alkyl represented by R_1, R_2, R_3, R_4, R_5, R_6, R_7, R_8, R_{10}, R_{20}, R_{31}, R_{32}, R_{41} or R_{42} is an alkyl group having 1 to 25 carbon atoms, which may be normal-chained, branched or cyclized. Examples of C_{1-25} alkyl include methyl, ethyl, propyl, isopropyl, cyclopropyl, butyl, isobutyl, tert-butyl, pentyl, isopentyl, cyclopentyl, hexyl, cyclohexyl, heptyl, 5-methylhexyl, cycloheptyl, octyl, 6-methylheptyl, nonyl, 7-methyloctyl, decyl and 8-methylnonyl, preferably C_{1-10} alkyl, and more preferably C_{1-6} alkyl. These alkyl groups may be substituted with other substituent(s), and may

comprise a heteroatom such as halogen, oxygen, sulfur, nitrogen or the like.

[0013] C_{2-25} alkenyl represented by R_1, R_2, R_3, R_4, R_5, R_6, R_7, R_8, R_{10}, R_{20}, R_{31}, R_{32}, R_{41} or R_{42} is an alkenyl group having 2 to 25 carbon atoms, which may be normal-chained, branched or cyclized. Examples of C_{2-25} alkenyl include vinyl, propenyl, 1,1-dimethyl-2-propenyl and 3-methyl-3-butenyl, preferably C_{2-10} alkenyl, and more preferably C_{2-6} alkenyl. These alkenyl groups may be substituted with other substituent(s), and may comprise a heteroatom such as halogen, oxygen, sulfur, nitrogen or the like.

[0014] C_{2-25} alkynyl represented by R_1, R_2, R_3, R_4, R_5, R_6, R_7, R_8, R_{10}, R_{20}, R_{31}, R_{32}, R_{41} or R_{42} is an alkynyl group having 2 to 25 carbon atoms, which may be normal-chained, branched or cyclized. Examples of C_{2-25} alkynyl include ethynyl, propynyl and butynyl, preferably C_{2-10} alkynyl, and more preferably C_{2-6} alkynyl. These alkenyl groups may be substituted with other substituent(s), and may comprise a heteroatom such as halogen, oxygen, sulfur, nitrogen or the like.

[0015] C_{1-25} alkoxy represented by R_1, R_2, R_3, R_4, R_5, R_6, R_7, R_8, R_{10}, R_{20}, R_{31}, R_{32}, R_{41} or R_{42} is an alkoxy group having 1 to 25 carbon atoms, which may be normal-chained, branched or cyclized. Examples of C_{1-25} alkoxy include methoxy, ethoxy, propoxy, isopropoxy, butoxy, isobutoxy, tert-butoxy, pentyloxy, isopentyloxy, cyclopentyloxy, hexyloxy, cyclohexyloxy, heptyloxy, 5-methylhexyloxy, cycloheptyloxy, octyl, 6-methylheptyloxy, nonyloxy, 7-methyloctyloxy, decyloxy and 8-methylnonyloxy, preferably C_{1-10} alkoxy, and more preferably C_{1-6} alkoxy. These alkoxy groups may be substituted with other substituent(s), and may comprise a heteroatom such as halogen, oxygen, sulfur, nitrogen or the like.

[0016] Aryl represented by R_1, R_2, R_3, R_4, R_5, R_6, R_7, R_8, R_{10}, R_{20}, R_{31}, R_{32}, R_{41} or R_{42} is a monocyclic or polycyclic aromatic hydrocarbon group, and examples include phenyl, naphthyl and anthranyl, but preferably phenyl. These aryl groups may be substituted with other substituent(s) such as C_{1-6} alkyl (preferably methyl, ethyl and propyl), C_{1-6} alkoxy, halogen, nitro, amino, carboxyl, hydroxy-C_{1-6} alkyl, hydroxyl or protected hydroxyl, and may comprise a heteroatom such as oxygen, sulfur, nitrogen or the like as a ring forming member.

[0017] Aralkyl represented by R_1, R_2, R_3, R_4, R_5, R_6, R_7, R_8, R_{10}, R_{20}, R_{31}, R_{32}, R_{41} or R_{42} is C_{1-6} alkyl substituted with the above aryl, and examples include benzyl, phenethyl, naphthylmethyl and anthranylmethyl, but preferably benzyl. These aralkyl groups may be substituted with other substituent(s) such as C_{1-6} alkyl (preferably methyl, ethyl and propyl), C_{1-6} alkoxy, halogen, nitro, amino, carboxyl, hydroxy-C_{1-6} alkyl, hydroxyl or protected hydroxyl, and may comprise a heteroatom such as oxygen, sulfur, nitrogen or the like as a ring forming member.

[0018] The examples of substituents in substituted amino represented by R_1, R_2, R_3, R_4, R_5, R_6, R_7, R_8, R_{10}, R_{20}, R_{31}, R_{32}, R_{41} or R_{42} include C_{1-6} alkyl, C_{1-6} alkoxy, halogen, carboxyl, hydroxy-C_{1-6} alkyl, hydroxyl or protected hydroxyl.

[0019] In the above formula (I), R_{10} and any of R_3, R_{31} and R_{32} may form a ring, and R_{20} and any of R_4, R_{41} and R_{42} may form a ring. In the above formula (II), R_2 and R_3 may form a ring, and R_4 and any of R_5, R_6, R_7 and R_8 may form a ring.

[0020] As C_{2-25} alkenyl represented by R_7 or R_8, an alkenyl group corresponding to an isoprene unit consisting of 5 carbon atoms, that is, 1,1-dimethyl-2-propenyl or 3-methyl-3-butenyl, and an alkenyl group consisting of two or more isoprene units, preferably at most 3 isoprene units (up to 15 carbon atoms) are desirable.

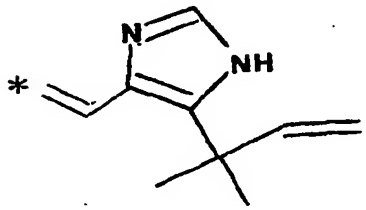
[0021] Substituents appearing in the above formulas (I) and (II) may have a protecting group capable of decomposing *in vivo*. Among these protecting groups, as a protecting group for an amino group for example, there may be used a protecting group having the binding form such as acid amide, carbamate and the like which are described in *Drug Development* vol. 13, "Drug Delivery Systems" edited by Hitoshi SEZAKI, Hirokawa Shoten (July 1989), page 116, Table 2. 29, but an acyl such as acetyl derived from fatty acid is preferable.

[0022] The double bond of a compound shown in the above formulas (I) or (II) may be either in Z configuration or in E configuration, but preferably in Z configuration.

[0023] In the case where (B1) and/or (B2) is a carbon-carbon double bond, the above substituent binding to the above carbon-carbon double bond becomes a corresponding divalent group. For example, methyl becomes methylene, and benzyl becomes phenylmethylene (benzylidene).

[0024] Among possible compounds represented by the above formula (I), a compound wherein each of X_1 and X_2 is oxygen, each of Y_3 and Y_4 is -NH-, R_{10} is benzyl, each of (B1) and (B2) is a carbon-carbon double bond, and R_{20} is isobutyl (the common name: albonoursin, the compound name: 3-(Z)-benzylidene-6-(Z)isobutylidene-2, 5-piperazine dione) refers to the known antitumor agent described in Fukushima *et al.*, *J. Antibiotics*, Vol.26, pp.175, 1973) and the known pronuclear fusion inhibitory agent described in *A* Summary *of the Symposium on the Chemistry of Natural Products*, P51, 1989, and these two agents are excluded from the cell division inhibitor of the present invention. In addition, a compound (tetradehydrocyclo (Phe-Phe)) wherein each of X_1 and X_2 is oxygen, each of Y_3 and Y_4 is -NH-, each of R_{10} and R_{20} is benzyl, and each of (B1) and (B2) is a carbon-carbon double bond refers to the known sea urchin embryo division inhibitory agent described in *A Summary of the Symposium of the Society for Actinomycetes Japan*, P42, 1999, and this agent is excluded from the cell division inhibitor of the present invention. Furthermore, a compound (the common name: phenylahistin, the compound name: 3-{[5-(1,1-dimethyl-2-propenyl)imidazole-4-yl] methylene}-6-benzylpiperazine-2,5-dione) wherein each of X_1 and X_2 is oxygen, each of Y_3 and Y_4 is -NH-, R_{10} is

benzyl, (B1) is a carbon-carbon single bond, (B2) is a carbon-carbon Z double bond, and R_{20} is a group shown in the following formula (a):



(a)

(wherein * represents a bonding position)

is the known cell division inhibitor disclosed in Japanese Patent Application Laying-Open (kokai) No. 10-130266 and so on, and this agent is excluded from the cell division inhibitor of the present invention. Generally, a compound wherein, in the above formula (I), each of X_1 and X_2 is independently oxygen or sulfur, Y_3 is $-NR_3-$, Y_4 is $-NR_4-$ (herein R_3 and R_4 are defined as with stated above), R_{10} is substituted or unsubstituted benzyl, (B1) is a carbon-carbon single bond, (B2) is a carbon-carbon Z double bond, and R_{20} is substituted or unsubstituted imidazole-4-ylmethylene is excluded from those used for the cell division inhibitor of the present invention.

[0025] Further, in the above formula (I), a compound wherein (B1 is a carbon-carbon double bond and (B2) is a carbon-carbon single bond or a carbon-carbon double bond is preferable, and a compound wherein each of (B1) and (B2) is a carbon-carbon double bond is more preferable.

[0026] Preferable examples of compounds, shown in the above formulas (I) and (II) include 3-(imidazole-4-ylmethylene)-6-(phenylmethylene)piperazine-2,5-dione; 3-[(5-methylimidazole-4-yl)methylene]-6-(phenylmethylene)piperazine-2,5-dione; 3-[(5-ethylimidazole-4-yl)methylene]-6-(phenylmethylene)piperazine-2,5-dione; 3-[(5-butylimidazole-4-yl)methylene]-6-(phenylmethylene)piperazine-2,5-dione; 3-[(5-pentylimidazole-4-yl)methylene]-6-(phenylmethylene)piperazine-2,5-dione; 3-{[5-(1,1-dimethyl-2-propenyl)imidazole-4-yl]methylene]-6-(phenylmethylene) piperazine-2,5-dione.

[0027] Pharmaceutically acceptable salt of a compound shown in the above formula (I) or (II) is ordinary organic or inorganic atoxic salt. In the case where the above compound is a basic substance, the salts that are preferably used are hydrochloride, hydrobromide, sulfate, nitrate, acetate, methanesulfonate and toluenesulfonate, and in the case where the compound is an acidic substance, the salt that is preferably used is a salt with inorganic base including alkali metallic salt (e.g. sodium salt, potassium salt etc.) and alkali-earth metallic salt (e.g. calcium salt, magnesium salt etc.) The term "pharmaceutically acceptable" in the present specification means that the salt is not only acceptable in medical agents, veterinary agents, agricultural chemicals, antimicrobial agents, insecticides etc., but also in a field comprising reagents used for study purposes.

[0028] The cell division inhibitor of the present invention can be used for the purpose of inhibiting the cell division, cell cycle and pronuclear fusion between a female nucleus and a male nucleus of a procaryote or an eucaryote. Specifically, the cell division inhibitor of the present invention is useful as an antimicrobial agent, agricultural chemical, veterinary agent, insecticide, medical agent and reagent for study purposes. Furthermore, among medical agents, it is particularly useful as an antitumor agent. The cell division inhibitor of the present invention is effective in a pathological condition wherein cell divisions are disorderly repeated. It is particularly useful for cancers, and also effective in a pathological condition appearing in a certain type of autoimmune disease, chronic articular rheumatism etc., where a certain type of cell continues to grow disorderly.

[0029] Furthermore, the antitumor agent of the present invention can comprise other pharmaceutically effective ingredients, i.e., other antitumor agents as necessary as well as the above active ingredients in order to treat various diseases. When the antitumor agent takes the form of granule, fine granule, powder, tablet or capsule, it is preferable that the antitumor agent comprises 5-80 weight % of the above active ingredients. When the antitumor agent takes a liquid form, it is preferable that the antitumor agent comprises 1-30 weight % of the above active ingredients. Further, when the antitumor agent is used as an injection among parenteral agents, it is preferable that the inhibitor comprises 1-10 weight % of the above active ingredients.

[0030] For use in oral administration, the applied dose of the above active ingredients is preferably 0.1mg to 1g per day per adult. However, depending on the age, body weight, symptom etc. of a patient, the dose can be changed as appropriate. The antitumor agent of the present invention can be administered once per day, but also it can be dividedly administered twice or three times at regular time intervals. When it is used as an injection, the applied dose of the above active ingredients is preferably 1 to several hundreds of milligrams per administration per adult. Moreover, it is possible to administer 1-3 times per day or once every 2 or 3 days by injection, or to administer sustainably by drip

infusion or the like.

[0031] As a substrate of the dehydrogenase of the present invention comprises a compound wherein, in the above formula (I), at least one of (B1) and (B2) is a carbon-carbon single bond, or a compound wherein, in the above formula (II), (B2) is a carbon-carbon single bond can be used, but preferably a compound wherein, in the above formula (I), each of X_1 and X_2 is oxygen, Y_3 is $-NR_3-$, and Y_4 is $-NR_4-$ (herein R_3 and R_4 are defined as with stated above), more preferably a compound wherein, in the above formula (I), each of X_1 and X_2 is oxygen, and each of Y_3 and Y_4 is -NH- is used, and further more preferably a cyclic dipeptide wherein two amino acids of L form condense to form a diketopiperazine ring or substituted compounds thereof is used. Examples of the above condensing amino acids preferably include cyclic (aromatic) amino acids such as phenylalanine, histidine, tryptophan and tyrosine. Examples of substituents in the substituted compounds of the above cyclic dipeptide include halogen, C_{1-25} alkyl, C_{2-25} alkenyl, C_{2-25} alkynyl, C_{1-25} alkoxy, aralkyl, hydroxyl, amino, nitro and aryl. These substituents may be substituted with other substituent(s), and a part of the carbon chain may be branched or cyclized, may comprise a heteroatom, may mutually form a ring, and may have a protecting group capable of decomposing *in vivo*. The substituents include preferably C_{2-6} alkyl or C_{2-6} alkenyl, more preferably 1,1-dimethyl-2-propenyl.

[0032] The majority of compounds used as the substrate stated above are the known compounds (Japanese Patent Application Laying-Open (kokai) No. 10-130266; Kanoh *et al., Bioorganic & Medicinal Chemistry Letters*, Vol.7, No. 22, pp.2847-2852, 1997; Kanoh *et al., Bioscience Biotechnology Biochemistry*, Vol.63, No.6, pp.1130-1133, 1999; Kanoh *et al., Bioorganic & Medicinal Chemistry*, Vol.7, pp.1451-1457, 1999), and these compounds are available. Other compounds can be produced by the same methods as those described in Kopple *et al., The Journal of Organic Chemistry*, Vol.33, pp.862-864, 1968 or Nitecki *et al., The Journal of Organic Chemistry*, Vol.33, pp.864-866, 1968.

[0033] The dehydrogenase of the present invention includes molecules having a variety of molecular weights, but one whose molecular weight is 700-800 kDa is preferable.

[0034] The present invention can use, as a coenzyme of the dehydrogenase, synthetic compounds such as dichlorophenolindophenol (DCIP), phenazine methosulfate (PMS), ferricyanide, tetramethylphenylenediamine and quinones, as well as natural compounds such as nicotin adenine dinucleotide (NAD), nicotin adenine dinucleotide phosphate (NADP), flavine adenine dinucleotide (FAD), flavin mononucleotide (FMN), pyrroloquinoline quinone (PQQ) and cytochromes. However, among them, FMN, PQQ, cytochromes, DCIP, PMS, ferricyanide, tetramethylphenylenediamine and quinones are preferable, and DCIP and/or PMS are further preferable.

[0035] The dehydrogenase of the present invention may be obtained from any organism, but ones derived from microorganisms such as bacteria, actinomycetes and filamentous fungi are preferable, ones from actinomycetes are more preferable, and ones from *Streptomyces albulus* are even more preferable.

[0036] The dehydrogenase from *Streptomyces albulus* has the following physicochemical properties:

(i) Function: The dehydrogenase from *Streptomyces albulus* acts to convert a carbon-carbon single bond on the position 3 or 6 into a carbon-carbon double bond.

(ii) Substrate specificity: The dehydrogenase from *Streptomyces albulus* converts phenylahistin into dehydrophenylahistin, and converts cyclophenylalanylhistidyl into dehydrocyclophenylalanylhistidyl or tetradehydrocyclophenylalanylhistidyl.

(iii) Optimum pH: 8.3

(iv) pH stability: stable at 7.0-9.0

(v) Optimum temperature: 60°C

(vi) Heat stability: stable at 20-70°C, deactivated at 80° C

(vii) Molecular weight: 700kDa-800kDa

[0037] The dehydrogenase of the present invention may be used not only as a natural tissue or cell, but also as a cell-free extract or enzyme solution obtained by partially or fully purifying the cell-free extract. The dehydrogenase may be purified according to the common enzyme purification method. Also, the multi-step reactions may be carried out at one time by mixing other enzymes.

[0038] The dehydrogenase of the present invention can produce a compound wherein, in the above formula (I), at least one of (B1) and (B2) is a carbon-carbon double bond or a compound wherein, in the above formula (II), (B2) is a carbon-carbon double bond, by using as a substrate a compound wherein, in the above formula (I), at least one of (B1) and (B2) is a carbon-carbon single bond, or a compound wherein, in the above formula (II), (B2) is a carbon-

carbon single bond. And these compounds are useful as a cell division inhibitor or an antitumor agent.

[0039] Some examples are provided below to describe the present invention more specifically.

[0040] The active ingredient of the cell division inhibitor of the present invention is a substance wherein, in the above formula (I), at least one of (B1) and (B2) is a carbon-carbon double bond, and representative examples include substituted or non-substituted dehydrodiketopiperazines, substituted or non-substituted tetradehydrodiketopiperazines, substituted or unsubstituted dehydro-cyclic dipeptide, substituted or unsubstituted tetradehydro-cyclic dipeptide, particularly substituted or unsubstituted dehydrocyclophenylalanylhistidyl or tetradehydrocyclophenylalanylhistidyl represented by the above formula (II), and further particularly dehydrophenylahistin.

[0041] As an example, the method of producing dehydrophenylahistin is provided below, but needless to say, the present invention is not limited thereto.

[0042] The method of collecting a novel compound dehydrophenylahistin by culturing an actinomycete, for example, *Streptomyces albulus* KO23 (which was deposited with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology (Higashi 1-1-3, Tsukuba-shi, Ibaragi-ken, Japan) under accession No. FERM BP-6994 on January 14, 2000), preparing a dehydrogenase from the culture, and allowing it to act on phenylahistin is specifically described later. But, as a dehydrogenase, either a purified enzyme or a natural cell extract may be used. Generally, the dehydrogenase can be prepared according to the method of culturing an actinomycete belonging to the genus *Streptomyces*. After the culture, in order to purify the dehydrogenase of the present invention from the culture solution or prepare a cell extract containing the enzyme activity, generally a common method used to purify the enzyme derived from microorganism can be applied as appropriate. For example, methods such as ultrasonic disintegration, centrifugation, salting out, dialysis, various ion exchange resin methods, nonionic adsorption method, chromatography including gel filtration chromatography, high performance liquid chromatography, crystallization or freeze-drying can be applied separately, in combination as appropriate, or repeatedly.

[0043] The method of carrying out dehydrogenation reaction by using an enzyme solution or cell extract prepared as above is specifically described in the Examples later, but the fact is that an enzyme solution and a substrate phenylahistin thereof are mixed to react in buffer such as a phosphate buffer. If necessary, it is possible to add an organic solvent to the reaction solution.

[0044] In order to purify and isolate dehydrophenylahistin from the above reaction solution, generally a common method of isolating/purifing organic compounds is applied as appropriate. For example, methods such as various ion exchange resin methods and nonionic adsorption methods; gel filtration chromatography, chromatography with adsorbents including activated carbon, alumina, silica gel etc., and high performance liquid chromatography; crystallization; vacuum concentration; or freeze-drying can be applied separately, in combination as appropriate, or repeatedly.

[0045] Dehydrophenylahistin produced by the above method has cell division inhibitory activity, as disclosed in Examples later. The usage, dosage form and applied dose (usage) of the cell division inhibitor of the present invention comprising dehydrophenylahistin as an active ingredient are determined as appropriate depending on the intended use. For example, in the case of the antitumor agent of the present invention comprising dehydrophenylahistin as an active ingredient, it may be administered either orally or parenterally. Examples of the dosage forms include oral preparations such as a tablet, powder, capsule, granule, extract and syrup, or parenteral preparations such as an injection or suppository. These formulations are produced using pharmaceutically acceptable additives such as an excipient or binder according to known methods. The applied dose of the antitumor agent containing the above dehydrophenylahistin as an active ingredient depends on the age, body weight, susceptibility, and symptoms of a patient. However, the effective amount is generally about 0.1mg to 1g per day per adult, and it is also possible to administer just once per day or devidedly several times per day. Furthermore, a dose beyond the above normal limits may be also administered as needed.

[0046] When the agent is used as a reagent for a biochemical examination, the development of the cell cycle is inhibited at the G2/M period, if the agent is dissolved in an organic solvent or hydrous organic solvent and administered directly to various cultured cell systems. Examples of the applicable organic solvents include methanol, dimethylsulfoxide etc. Examples of the dosage forms include solid agents such as powder or granule, liquid agents dissolved in organic solvent or hydrous organic solvent, and the like. Generally, an effective amount of the cell division inhibitor comprising the above dehydrophenylahistin as an active ingredient is 0.01-100μg/mL, but the appropriate amount depends on the type of cultured cell system or intended use. Further, an amount beyond the above normal limits may be also administered as needed.

[0047] This specification includes part or all of the contents as disclosed in the specification of Japanese Patent Application No. 2000-9370 which is a priority document of the present application.

Best Mode for Carrying Out the Invention

[0048] The present invention will be further described in the following examples. In the following examples, cyclo (A_1-A_2), which is a cyclic dipeptide formed by condensation of two amino acids A_1 and A_2 into a diketopiperazine ring,

is designated CA_1A_2 (A_1 and A_2 represent amino acids in single-letter notation, respectively). All of the cyclic dipeptides CA_1A_2 are LL-isomers unless otherwise specified. A D-amino acid is designated, for example, DA_1, if necessary. Further, dehydro-peptides are designated Δ, so that $C\Delta A_1A_2$ represents cyclo(ΔA_1-A_2), $CA_1\Delta A_2$ represents cyclo(A_1-ΔA_2), $C\Delta A_1\Delta A_2$ represents cyclo(ΔA_1-ΔA_2), and ΔCA_1A_2 represents a mixture of $C\Delta A_1A_2$, $CA_1\Delta A_2$ and $C\Delta A_1\Delta A_2$. Furthermore, PLH represents phenylahistin.

Example 1

(1) Phenylahistin was prepared as follows.

[0049] Phenylahistin-producing bacterial cells (*Aspergillus ustus* NSC-F038, which was deposited with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology (Higashi 1-1-3, Tsukuba-shi, Ibaragi-ken, Japan) under accession No. FERM P-15830 on September 3, 1996), were inoculated onto five spots on a solid medium (20 ml per 9 cm dish) which contains 0.5% glucose, 2% glycerol, 0.2% yeast extract, 2% Pharmamedia (cottonseed cake), 0.25% sodium chloride and 1.5% agar (pH 6.5). The cells were then cultured at 26 °C for 7 days in the dark to obtain a spore suspension. The resulting spore suspension (0.1 ml) was inoculated onto each of 400 dishes containing 20 ml of the above solid medium, and then cultured at 26 ° C for 8 days in the dark. The resulting culture was crushed using a mixer, and after addition of 8L ethyl acetate, was allowed to stand for 2 days then extracted. The collected ethyl acetate layer was concentrated under vacuum to obtain 15 g brown syrup. This syrup was dissolved in 20 ml ethyl acetate and applied to a silica gel column (8 cm in diameter, 20 cm in length) prepared with 1:6 acetone-ethyl acetate, followed by elution with 1:6 acetone-ethyl acetate. The eluted solution was fractionated into 500 ml fractions in order of elution. Phenylahistin was contained in the fifth to tenth fractions, which were then concentrated under vacuum to obtain 4.7 g dark brown powder in total. This dark brown powder was dissolved in 10 ml chloroform and applied to a silica gel column (4 cm in diameter, 30 cm in length) prepared with chloroform, followed by elution with 500 ml chloroform and then 50:1 chloroform-methanol. The compound of interest was eluted with 50:1 chloroform-methanol to obtain 1.05 g brown powder in total. After addition of 100 ml ethyl acetate, this brown powder was mixed well and allowed to stand for 2 days to separate out 628 mg phenylahistin as white powder.

(2) Culture of *Streptomyces albulus* KO23 and preparation of a cell-free extract were carried out as follows,

[0050] Ten milliliters of sterilized water containing 50-200 µl surfactant (Triton X-100) was added to and mixed with a slant on which gray spores had formed well, thereby obtaining a spore suspension. This suspension was diluted 1000-fold in a culture medium and cultured under the following conditions. The culture medium had the composition shown in Table 1.

Table 1

KP medium composition (g/L)	
Glucose	15
Glycerol	10
Polypepton	10
Beef extract	10
$CaCO_3$	4
	pH 7.3

[0051] Table 2 shows the culture conditions.

Table 2

Culture conditions	
Pre-culture in 200 ml Erlenmeyer flask	
KP medium	40 ml
Culture period	24 hours
Rotation speed	180 rpm
Temperature	28 °C

Table 2 (continued)

Culture conditions	
Main culture in 5 L jar fermenter	
KP medium	3 L
Antifoaming agent (Antiform AFI emulsion)	10 g per 3 L
Culture period	48 hours
Rotation speed	300 rpm
Ventilation volume	2 L per 3 min
Temperature	28°C

[0052] The cell-free extract was prepared as follows.

[0053] The culture solution (40 ml) was centrifuged at 20,000 × g for 15 min at 4 °C to collect the cells. These cells were suspended in 40 ml physiological saline, and then centrifuged again at 20,000 × g for 15 min at.4 °C to wash the cells. These cells were suspended in 7.3 ml sodium phosphate buffer (10 mM, pH 8.0), followed by ultrasonication (150 W, 1.5 min, KUBOTA INSONATOR 201M). The resulting solution was centrifuged at 20,000 × g for 15 min at 4 °C to obtain the supernatant as a cell-free extract.

(3) Conversion reaction of phenylahistin into dehydrophenylahistin and purification of the reaction product were carried out as follows.

[0054] The reaction mixture had the composition shown in Table 3.

Table 3

Reaction mixture composition	
Phenylahistin	0.5 mg/ml
Dimethyl sulfoxide	10 % (v/v)
Sodium phosphate buffer (pH 8.0)	9 mM
Cell-free extract	0.145 units/ml
Temperature	50 °C

[0055] The above reaction mixture (100 ml) was divided into 200 ml Erlenmeyer flasks to contain 20 ml reaction mixture in each flask. The reaction was carried out at 160 strokes/min for 24 hours, followed by centrifugation at 20,000 × g for 15 min at 4 °C to obtain a yellow precipitate. This precipitate was dissolved in 55 ml methanol, and then centrifuged again at 20,000 × g for 15 min at 4 °C. The resulting supernatant was vacuumed-concentrated and dried to a solid, followed by recrystallization from methanol, thereby obtaining 5.58 mg dehydrophenylahistin as a yellow needle crystal.

[0056] The resulting dehydrophenylahistin has the following physicochemical data:

EIMS m/z: 348 (M^+, 100), 133 (25), 160 (17), 260 (16).

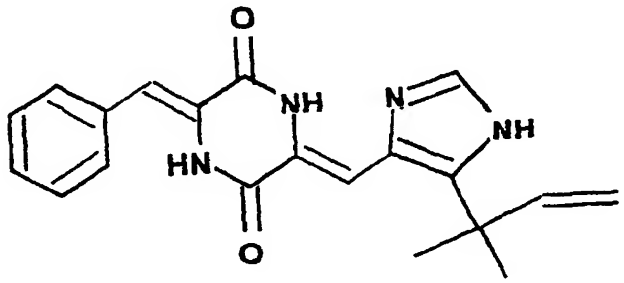
UV (MeOH) lmax, nm (e): 205 (16600), 363 (35300).

^{1}H-NMR (500 MHz, $CDCl_3$):

δ 1.51, 6H, s
δ 5.16, 1H, d (J=17.4)
δ 5.20, 1H, d (J=10.7)
δ 6.03, 1H, dd (J=10.7, 17.4)
δ 6.96, 1H, s
δ 6.98, 1H, s
δ 7.32, 1H, d (J=7.0)
δ 7.37, 2H, d (J=7.3)
δ 7.43, 2H, dd (J=7.0, 7.3)
δ 7.57, 1H, s
δ 8.04, 1H, s
δ 9.06, 1H, br s

δ 12.23, 1H, s

[0057] The resulting product was identified as (Z, Z)-dehydrophenylahistin based on NOE observed between a proton of diketopiperazine (δ 8.04, 1H, s) and protons of phenyl group (d 7.43, 2H, dd (J=7.0, 7.3)). It has the following structural formula (III):



(III)

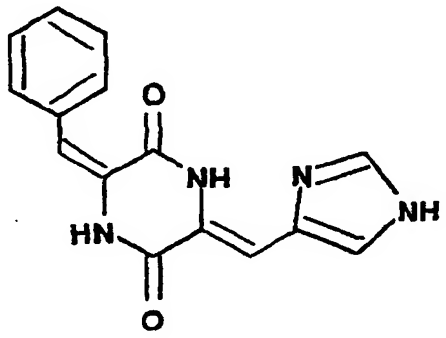
Example 2

[0058] Dehydro-products of cyclophenylalanylhistidyl (CFH) were prepared from CFH through dehydrogenation as follows.

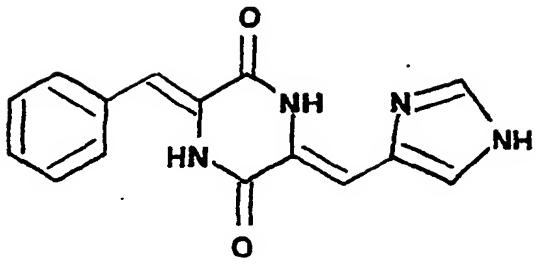
Table 4

Reaction mixture composition	
CFH	0.5 mg/ml
Dimethyl sulfoxide	10 % (v/v)
Sodium phosphate buffer (pH 8.0)	9 mM
Cell-free extract from Example 1	0.435 units/ml

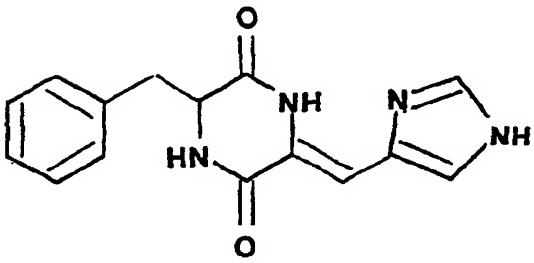
[0059] The reaction mixture (100 ml) shown in Table 4 was prepared and divided into five 20 ml Erlenmeyer flasks. The reaction was carried out in Reciprocal (160 strokes/min) at 50 °C for 24 hours. After 24 hours, the reaction mixture was centrifuged at 20,000 × g for 15 min at 4 °C to obtain the supernatant. This supernatant was extracted with ethyl acetate, and then purified by HPLC (Waters 600 Controller, 486 Tunable Absorbance Detector, 616 Pump, Inertsil ODS-3 column ϕ 20 mm × 250 mm, 60% methanol as a solvent, flow rate of 10 ml/min, UV detection at 256 nm), thereby obtaining three dehydro-products at retention times of 3.9 min, 9.1 min and 11.6 min. Instrumental analysis indicates that the product eluted at 9.1 min is E-tetradehydrocyclophenylalanylhistidyl (CE-ΔFΔH) of the formula (IV), the product eluted at 11.6 min is Z-tetradehydrocyclophenylalanylhistidyl (CZ-ΔFΔH) of the formula (V), and the product eluted at 30.1 min is dehydrocyclophenylalanylhistidyl (CFΔH) of the formula (VI).



(IV)



(V)



(VI)

[0060] The compound (V) has the following physicochemical data:

EIMS m/z: 280 (M^+, 100), 107 (36), 279 (29), 281 (18).
UV (MeOH) lmax, nm(e): 205 (14800), 257 (6500), 351 (27100).
^{1}H-NMR (500 MHz, $CDCl_3$):
δ 6.77, 1H, s
δ 7.02, 1H, s
δ 7.22, 1H, m
δ 7.33, 1H, t (J=7.3)
δ 7.37, 2H, d (J=7.3)
δ 7.43, 2H, dd (J=7.3, 7.3)
δ 7.75, 1H, s
δ 8.09, 1H, s
δ 9.30, 1H, br s
δ 11.91, 1H, s

Example 3

[0061] A variety of dehydrodiketopiperazines were prepared from different diketopiperazines as substrates through dehydrogenation reactions using the enzyme of the present invention as follows.

Table 5

Reaction mixture composition	
Dimethyl sulfoxide (DMSO)	10% (v/v)
Sodium phosphate buffer (pH 8.0)	5.2 mM
Dichlorophenolindophenol (DCIP)	80 μM
Phenazine methosulfate (PMS)	120 μM
Cell-free extract from Example 1	q.s.
Substrate	0.5 mM
Total	0.5 ml

[0062] The reaction mixture shown in Table 5 was used for the dehydrogenation reaction at 37 °C. The reaction product was analyzed by HPLC and detected by UV absorbance at 256 nm. This method provided the following dehydro-products: ΔCAF, ΔCFF, ΔCFG, ΔCFH, ΔCFL, CΔFL, CFΔL, ΔCFS, ΔCFV, ΔCFW, ΔCLW, ΔCLY, ΔCVY, ΔCWW, ΔCWY, ΔCDWY (W residue is D-form), and ΔPLH.

Example 4

[0063] A variety of dehydrodiketopiperazines were prepared from different diketopiperazines as substrates through dehydrogenation reactions using the enzyme of the present invention as follows.

Table 6

Reaction mixture composition	
Dimethyl sulfoxide (DMSO)	10% (v/v)
Sodium phosphate buffer (pH 8.0)	5.2 mM
Cell-free extract from Example 1	q.s.
Substrate	0.5 mg/ml
Total	0.5 ml

[0064] The reaction mixture shown in Table 6 was used for the dehydrogenation reaction at 37 °C. The reaction product was analyzed by HPLC and detected by a photodiode array detector (multi-channel UV, 220 nm to 400 nm). This method provided the following dehydro-products:
ΔCAH, ΔCAW, ΔCAY, ΔCD(OMe)D(OMe), ΔCDF, ΔCFG, ΔCFS, ΔCFV, ΔCFW, ΔCGL, ΔCGW, ΔCGY, ΔCHH, ΔCHW, ΔCHY, ΔCLP, ΔCLW, ΔCLY, ΔCMM, ΔCSY, ΔCVW, ΔCWW, ΔCWY, ΔCDWY (W residue is D-form), and ΔCD(OEt)G, wherein D(OMe) represents an aspartic acid having a methylated carboxyl group on its side chain (γ-position), and D (OEt) represents an aspartic acid having a ethylated carboxyl group on its side chain (γ-position).

Example 5

[0065] A variety of dehydrodiketopiperazines were prepared from different diketopiperazines as substrates through dehydrogenation reactions using the enzyme of the present invention as follows.

[0066] The reaction procedures as described in Example 3 were repeated and an amount of dehydrogenation by the enzyme was determined based on a change in absorbance at 600 nm due to coenzyme. Table 7 shows the amount of dehydrogenation by the enzyme (i.e., a change in absorbance) for each substrate, which is expressed as a relative value (an absorbance for CFL was set to 100).

Table 7

Amount of dehydrogenation of each substrate by enzyme	
Substrate	Amount of dehydrogenation
CFL	100
CFH	44
CMM	27
CEE	14
CLY	14
CDD	14

Example 6

[0067] Dehydrogenase derived from *Streptomyces albulus* KO23, which requires diketopiperazine as its substrate, was purified according to the procedures as described in Example 1.

[0068] *Streptomyces albulus* KO23 was cultured in a mini jar containing 3 L culture medium to obtain 167.12 g of the cells. The cell-free extract was prepared from these cells as follows.

Table 8

Preparation of cell-free extract				
Conversion activity (units/ml)	Protein (A_{280}) (mg/ml)	Specific activity (units/mg)	Liquid volume (ml)	Total activity (units)
0.684	14.2	0.0482	382	261.3

[0069] The resulting extract was subjected to DEAE-Sephacel anion exchange column chromatography.

Column	DEAE-Sephacel ϕ 2.6 cm × 30 cm
Flow rate	1 ml/min
Fraction size	10 ml
Sample	113 ml cell-free extract

[0070] As a buffer, 10 mM sodium phosphate buffer (pH 8.0) containing 0.1 mM DTT was used. After the sample was adsorbed to the column, the column was washed with 360 ml buffer, and then eluted stepwise with 400 ml buffer containing 0.1 M NaCl, 410 ml buffer containing 0.3 M NaCl, and 600 ml buffer containing 0.5 M NaCl, thereby obtaining the following active fractions.

Table 9

Purification by DEAE-Sephacel anion exchange column chromatography					
Fraction	Conversion activity (units/ml)	Protein (A_{280}) (mg/ml)	Specific activity (units/mg)	Liquid volume (ml)	Total activity (units)
50-56	0.179	1.42	0.126	70	12.5
57-71	0.240	2.56	0.0781	152	30.4

[0071] Fractions 50-56 having a higher specific activity were subjected to the subsequent Mono-Q column chromatography as follows.

Column	MonoQ HR 5/5
Flow rate	1 ml/min
Fraction size	0.6 ml
Sample	4 × 1 ml DEAE-Sephacel fractions 50-56 diluted 2-fold with buffer

[0072] As a buffer, 10 mM sodium phosphate buffer (pH 8.0) containing 0.1 mM DTT was used. After the sample was adsorbed to the column, the column was washed with the buffer for 4 minutes, and then eluted with 1 M NaCl-containing buffer using a linear gradient (25 min). The above procedures were repeated four times to obtain the following active fraction.

Table 10

Purification by MonoQ anion exchange column chromatography				
Conversion activity (units/ml)	Protein (A_{280}) (mg/ml)	Specific activity (units/mg)	Liquid volume (ml)	Total activity (units)
0.0201	0.0376	0.646	7.2	0.145

[0073] The above active fraction was subjected to gel filtration chromatography (Superose 12) as follows.

Column	Superose 12 HR 10/30
Flow rate	0.5 ml/min
Fraction size	0.25 ml
Sample	MonoQ active fraction concentrated to 225 µl

[0074] As a buffer, 10 mM sodium phosphate buffer (pH 8.0) containing 0.1 mM DTT and 0.3 M NaCl was used. Table 11 shows enzyme activity of each fraction. The most active fractions 13-16 were combined together and concentrated by ultrafiltration.

Table 11

Purification by Superose 12 gel filtration column chromatography			
Fraction	Conversion activity (units/ml)	Liquid volume (μL)	Total activity (units)
11, 12	0.0737	100	7.37
13, 14	0.253	45	11.4
15, 16	0.184	45	8.28
17, 18	0.0526	80	4.21

[0075] The above active fraction was subjected to gel filtration chromatography (TSK G3000SWXL) with Waters LC Modulel as follows.

Column	TSK GEL G3000SWXL
Flow rate	0.5 ml/min
Sample	Superose active fraction concentrated to 40 μl

[0076] As a buffer, 100 mM sodium phosphate buffer (pH 7.5) containing 0.1 mM DTT and 0.3 M NaCl was used. The resulting active fractions were combined together and concentrated by ultrafiltration. Table 12 shows enzyme activity of the combined and concentrated fraction.

Table 12

Purification by TSK G3000SWXL gel filtration chromatography		
Activity (units)	Protein(A_{280}) (mg/ml)	Specific activity (units/mg)
0.00224	0.00114	19.6

[0077] Table 13 shows enzyme activity in each step of the purification procedures and a final enzyme activity.

Table 13

Enzyme purification and specific activity			
Purification step	Enzyme activity (units)	Protein (mg)	Specific activity (units/mg)
Cell-free extract	0.734	15.2	0.0482
DEAE-Sephacel	0.119	0.946	0.126
Mono-Q	0.0644	0.120	0.537
Superose 12	0.00790	0.00799	0.989
TSK G3000SW	0.00224	0.000114	19.6

Example 7

[0078] The reaction mixture shown in Table 14 was used for the enzymatic reaction using the enzyme of the present invention. Various diketopiperazines were used as substrates. The resulting enzymatic reaction mixture was tested for its inhibitory activity against embryo division of *Temnopleurus toreumaticus* without any purification of the reaction product. The test was carried out as described in The Journal of Antibiotics, Vol. 52, p. 1017 (1999). However, stages at which the first cleavage division occurs vary among sea urchins, so that inhibition of the cleavage division was observed after one hour of fertilization in this test using *Temnopleurus toreumaticus*. Concentration of the substrate

added to the enzymatic reaction system was used as a criterion for inhibitor concentration because the reaction product was used for the test without any purification. The inhibition test for the embryo division of *Temnopleurus toreumaticus* started with the highest substrate concentration of 25 μg/ml, followed by serially diluted substrate concentrations. Table 15 shows the test results.

Table 14

Reaction mixture composition	
Dimethyl sulfoxide (DMSO)	10% (v/v)
Sodium phosphate buffer (pH 8.0)	5.2 mM
Cell-free extract from Example 1	q.s.
Substrate	0.5 mg/ml
Total	0.2 ml

Table 15

Inhibition test for cleavage division using the enzymatic reaction mixture	
	MIC (μg/ml)
CDF reaction product	>25 (80% inhibition at 25 μg/ml)
CFF reaction product	>25 (90% inhibition at 25 μg/ml)
CFV reaction product	25
CGL reaction product	>13 (70% inhibition at 13 μg/ml)
CHW reaction product	13
CLY reaction product	>13 (60% inhibition at 13 μg/ml)
CWY reaction product	6.3

Example 8

[0079] Physiological activity of each dehydrodiketopiperazine will be described below. Each dehydrodiketopiperazine was tested for its inhibitory activity against cleavage division of *Hemicentrotus pulcherrimus*, *Scaphechinus mirabilis* and *Temnopleurus toreumaticus* as a cell division inhibitory activity. The test was carried out as described in The Journal of Antibiotics, Vol. 52, p. 1017 (1999). However, stages at which the first cleavage division occurs vary among sea urchins, so that inhibition of the cleavage division was observed after 4 hours of fertilization in the tests using *Hemicentrotus pulcherrimus* and *Scaphechinus mirabilis,* and after one hour of fertilization in the test using *Temnopleurus toreumaticus*, respectively. Table 16 shows the test results.

Table 16

Inhibition test for cell division using dehydrophenylahistin and related compounds				
		MIC, μg/ml		
	Compound	*Scaphechinus mirabilis*	*Temnopleurus toreumaticus*	*Hemicentrotus pulcherrimus*
Example 1	dehydrophenylahistin	0.0061	0.0061	0.00038
Example 2	(Z,Z)-tetradehydro-CFH	1.6	1.6	0.78
Comparison 1	(-)-phenylahistin	1.6	0.2	0.39
Comparison 2	(+)-phenylahistin	> 13*	6.3	13
Comparison 3	albonoursin	> 13*	> 25*	6.3
Comparison 4	CFH	> 25*	> 25*	> 25*

* no activity at the indicated concentration

[0080] Dehydrophenylahistin has MIC of 0.0061 μg/ml for cell division of *Scaphechinus mirabilis* and *Temnopleurus toreumaticus*, and MIC of 0.00038 μg/ml for cell division of *Hemicentrotus pulcherrimus*, respectively. Dehydrophenylahistin exhibits 250-fold to 1000-fold inhibitory activity when compared with non-dehydrogenated (-)-phenylahistin. (Z,

Z)-tetradehydro-CFH obtained by dehydrogenation of CFH exhibits 15-fold or more inhibitory activity when compared with CFH. In any case, a variety of dehydrodiketopiperazines including dehydrophenylahistin and (Z,Z)-tetradehydro-CFH were shown to have the cell division inhibitory activity, indicating that the dehydrodiketopiperazines are useful as cell division inhibitors and antitumor agents.

Formulation example 1: Formulation for injection or drip infusion

[0081] One milligram of dehydrophenylahistin and 5g of glucose powder were aseptically distributed to each vial. Each vial was sealed under an inert gas such as nitrogen or helium, and then stored in a cool dark place. Before use, ethanol was added to each vial to dissolve its content, followed by addition of 100 ml 0.85% physiological saline to produce a formulation for intravenous injection. The resulting formulation is intravenously injected or infused in an amount of 10 to 100 ml per day depending on symptoms.

Formulation example 2: Formulation for injection or drip infusion

[0082] The procedures as described in Formulation example 1 were repeated to produce a formulation for intravenous injection containing 0.2 mg dehydrophenylahistin, which may be used for treatment of mild cases. The resulting formulation is intravenously injected or infused in an amount of 10 to 100 ml per day depending on symptoms.

Formulation example 3: Granules

[0083] One hundred milligrams of dehydrophenylahistin, 98 g of lactose and 1 g of hydroxypropylcellulose were mixed well, granulated by standard techniques, dried well and passed through a mesh, thereby obtaining granules suitable for packaging in a bottle or heat seal. The resulting granules are orally administered in an amount of 100 to 1000 mg per day depending on symptoms.

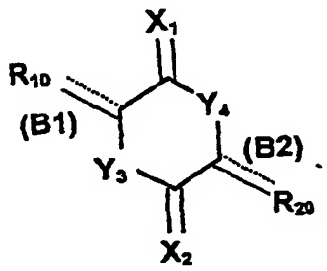
[0084] All publications, patents and patent applications cited herein are incorporated herein by reference in their entirety.

Industrial Applicability

[0085] The present invention provides a cell division inhibitor having stronger cell cycle inhibitory activity, particularly antitumor activity, and an enzyme usable for the production thereof.

Claims

1. A cell division inhibitor comprising, as an active ingredient, a compound of formula (I) or pharmaceutically acceptable salt thereof:

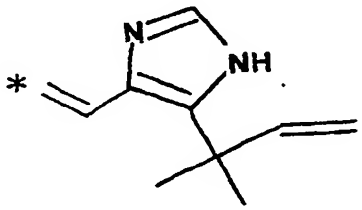


(I)

wherein

each of X_1 and X_2 is independently oxygen or sulfur;
Y_3 is oxygen, sulfur, $-NR_3-$ or $-CR_{31}R_{32}-$;
Y_4 is oxygen, sulfur, $-NR_4-$ or $-CR_{41}R_{42}-$;
R_{10} is halogen, C_{1-25} alkyl, C_{2-25} alkenyl, C_{2-25} alkynyl, C_{1-25} alkoxy, aralkyl, hydroxyl, amino, nitro or aryl, which may be substituted with other substituent(s), and a part of the carbon chain of R_{10} may be branched or

cyclized, or may comprise a heteroatom;
R_{20} is halogen, $C_{1\text{-}25}$ alkyl, $C_{2\text{-}25}$ alkenyl, $C_{2\text{-}25}$ alkynyl, $C_{1\text{-}25}$ alkoxy, aralkyl, hydroxyl, amino, nitro or aryl, which may be substituted with other substituent(s), and a part of the carbon chain of R_{20} may be branched or cyclized, or may comprise a heteroatom;
each of R_3 and R_4 is independently hydrogen, halogen, $C_{1\text{-}25}$ alkyl, $C_{2\text{-}25}$ alkenyl, $C_{2\text{-}25}$ alkynyl, $C_{1\text{-}25}$ alkoxy, aralkyl, hydroxyl, amino, nitro or aryl, which may be substituted with other substituent(s), and a part of the carbon chain may be branched or cyclized, or may comprise a heteroatom;
each of R_{31}, R_{32}, R_{41} and R_{42} is independently hydrogen, halogen, $C_{1\text{-}25}$ alkyl, $C_{2\text{-}25}$ alkenyl, $C_{2\text{-}25}$ alkynyl, $C_{1\text{-}25}$ alkoxy, aralkyl, hydroxyl, amino, nitro or aryl, which may be substituted with other substituent(s), and a part of the carbon chain may be branched or cyclized, or may comprise a heteroatom;
R_{10} and any of R_3, R_{31} and R_{32} may form a ring;
R_{20} and any of R_4, R_{41} and R_{42} may form a ring;
each of (B1) and (B2) independently represents a carbon-carbon single bond or a carbon-carbon double bond, wherein at least one represents a carbon-carbon double bond with E or Z configuration;
at least one of said groups may have a protecting group capable of decomposing *in vivo*, except in the case where each of X_1 and X_2 is oxygen, each of Y_3 and Y_4 is -NH-, R_{10} is benzyl, each of (B1) and (B2) is a carbon-carbon double bond, and R_{20} is isobutyl or benzyl, and in the case where each of X_1 and X_2 is oxygen, each of Y_3 and Y_4 is -NH-, R_{10} is benzyl, (B1) is a carbon-carbon single bond, (B2) is a carbon-carbon Z double bond, and R_{20} is a group shown in the following formula (a):



(a)

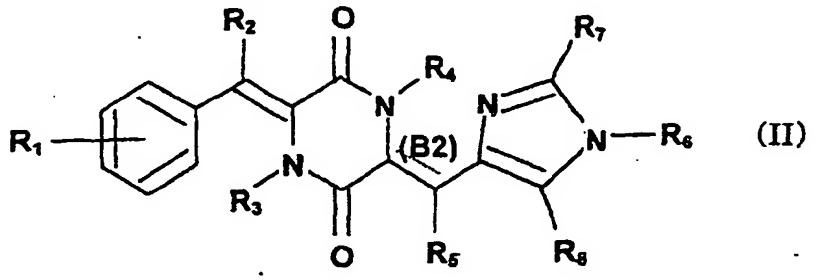
wherein * represents a bonding position.

2. The cell division inhibitor according to claim 1 wherein, in said formula (I), each of (B1) and (B2) is a carbon-carbon double bond.

3. The cell division inhibitor according to any one of claim 1 or 2 wherein, in said formula (I), each of X_1 and X_2 is oxygen, Y_3 is $-NR_3-$, and Y_4 is $-NR_4-$.

4. The cell division inhibitor according to claim 3 wherein, in said formula (I), each of Y_3 and Y_4 is -NH-.

5. A cell division inhibitor comprising, as an active ingredient, a compound of formula (II) or an E form thereof, or pharmaceutically acceptable salt thereof:



(II)

wherein

R_1 is hydrogen, halogen, $C_{1\text{-}25}$ alkyl, $C_{2\text{-}25}$ alkenyl, $C_{2\text{-}25}$ alkynyl, $C_{1\text{-}25}$ alkoxy, aralkyl, hydroxyl, amino, nitro

or aryl, which may be substituted with other substituent(s), and a part of the carbon chain of R_1 may be branched or cyclized, or may comprise a heteroatom, and further R_1 may be one atom or group, or at most 5 identical or different atoms or groups, and the atoms or groups may mutually form a ring;
R_2 is hydrogen, halogen, C_{1-25} alkyl, C_{2-25} alkenyl, C_{2-25} alkynyl, C_{1-25} alkoxy, aralkyl, hydroxyl, amino, nitro or aryl, which may be substituted with other substituent(s), and a part of the carbon chain of R_2 may be branched or cyclized, or may comprise a heteroatom;
each of R_3 and R_4 is independently hydrogen, halogen, C_{1-25} alkyl, C_{2-25} alkenyl, C_{2-25} alkynyl, C_{1-25} alkoxy, aralkyl, hydroxyl, amino, nitro or aryl, which may be substituted with other substituent(s), and a part of the carbon chain may be branched or cyclized, or may comprise a heteroatom;
R_5 is hydrogen, halogen, C_{1-25} alkyl, C_{2-25} alkenyl, C_{2-25} alkynyl, C_{1-25} alkoxy, aralkyl, hydroxyl, amino, nitro or aryl, which may be substituted with other substituent(s), and a part of the carbon chain of R_5 may be branched or cyclized, or may comprise a heteroatom;
R_6 is hydrogen, halogen, C_{1-25} alkyl, C_{2-25} alkenyl, C_{2-25} alkynyl, C_{1-25} alkoxy, aralkyl, hydroxyl, amino, nitro or aryl, which may be substituted with other substituent(s), and a part of the carbon chain of R_6 may be branched or cyclized, or may comprise a heteroatom;
each of R_7 and R_8 is independently hydrogen, halogen, C_{1-25} alkyl, C_{2-5} alkenyl, C_{2-25} alkynyl, C_{1-25} alkoxy, aralkyl, hydroxyl, amino, nitro or aryl, which may be substituted with other substituent(s), and a part of the carbon chain may be branched or cyclized, or may comprise a heteroatom;
R_2 and R_3 may form a ring;
R_4 and any of R_5, R_6, R_7 and R_8 may form a ring;
(B2) represents a carbon-carbon single bond or a carbon-carbon double bond;

at least one of said groups may have a protecting group capable of decomposing *in vivo*.

6. The cell division inhibitor according to claim 5 wherein, in said formula (II), (B2) is a carbon-carbon double bond.

7. The cell division inhibitor according to claim 6 wherein, in said formula (II), at least one of R_7 and R_8 is 1, 1-dimethyl-2-propenyl.

8. The cell division inhibitor according to any one of claims 1-7 which is an antitumor agent.

9. A dehydrogenase which has an activity to convert a compound represented by said formula (I) wherein at least one of (B1) and (B2) is a carbon-carbon single bond, or by said formula (II) wherein (B2) is a carbon-carbon single bond into a compound wherein said carbon-carbon single bond(s) is replaced with a carbon-carbon double bond(s).

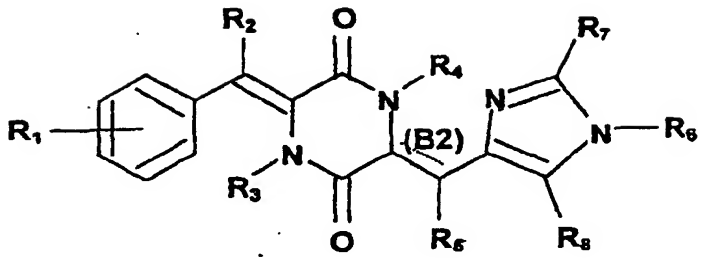
10. The dehydrogenase according to claim 9 whose molecular weight is 700-800 kDa.

11. The dehydrogenase according to claim 9 or 10 which is produced by *Streptomyces albulus*.

12. A method of producing the cell division inhibitor according to any one of claims 1-8, which comprises using, as a substrate, a compound represented by said formula (I) wherein at least one of (B1) and (B2) is a carbon-carbon single bond, or a compound represented by said formula (II) wherein (B2) is a carbon-carbon single bond, and converting said carbon-carbon single bond to a carbon-carbon double bond by use of a cell, cell-free extract or enzyme solution containing the dehydrogenase according to any one of claims 9-11.

13. The method according to claim 12 wherein the dehydrogenase of claim 11 is used.

14. A compound of formula (II) or an E form thereof, or pharmaceutically acceptable salt thereof:



(II)

wherein

R_1 is hydrogen, halogen, C_{1-25} alkyl, C_{2-25} alkenyl, C_{2-25} alkynyl, C_{1-25} alkoxy, aralkyl, hydroxyl, amino, nitro or aryl, which may be substituted with other substituent(s), and a part of the carbon chain of R_1 may be branched or cyclized, or may comprise a heteroatom, and further R_1 may be one atom or group, or at most 5 identical or different atoms or groups, and the atoms or groups may mutually form a ring;
R_2 is hydrogen, halogen, C_{1-25} alkyl, C_{2-25} alkenyl, C_{2-25} alkynyl, C_{1-25} alkoxy, aralkyl, hydroxyl, amino, nitro or aryl, which may be substituted with other substituent(s), and a part of the carbon chain of R_2 may be branched or cyclized, or may comprise a heteroatom;
each of R_3 and R_4 is independently hydrogen, halogen, C_{1-25} alkyl, C_{2-25} alkenyl, C_{2-25} alkynyl, C_{1-25} alkoxy, aralkyl, hydroxyl, amino, nitro or aryl, which may be substituted with other substituent(s), and a part of the carbon chain may be branched or cyclized, or may comprise a heteroatom;
R_5 is hydrogen, halogen, C_{1-25} alkyl, C_{2-25} alkenyl, C_{2-25} alkynyl, C_{1-25} alkoxy, aralkyl, hydroxyl, amino, nitro or aryl, which may be substituted with other substituent(s), and a part of the carbon chain of R_5 may be branched or cyclized, or may comprise a heteroatom;
R_6 is hydrogen, halogen, C_{1-25} alkyl, C_{2-25} alkenyl, C_{2-25} alkynyl, C_{1-25} alkoxy, aralkyl, hydroxyl, amino, nitro or aryl, which may be substituted with other substituent(s), and a part of the carbon chain of R_6 may be branched or cyclized, or may comprise a heteroatom;
each of R_7 and R_8 is independently hydrogen, halogen, C_{1-25} alkyl, C_{2-25} alkenyl, C_{2-25} alkynyl, C_{1-25} alkoxy, aralkyl, hydroxyl, amino, nitro or aryl, which may be substituted with other substituent(s), and a part of the carbon chain may be branched or cyclized, or may comprise a heteroatom;
R_2 and R_3 may form a ring;
R_4 and any of R_5, R_6, R_7 and R_8 may form a ring;
(B2) represents a carbon-carbon single bond or a carbon-carbon double bond;

at least one of said groups may have a protecting group capable of decomposing *in vivo*.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP00/06807

A. CLASSIFICATION OF SUBJECT MATTER
Int.Cl[7] C07D403/06, A61K31/496, A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int.Cl[7] C07D403/06, A61K31/496, A61P35/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CA(STN)
REGISTRY(STN)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JP, 10-130266, A (Nippon Steel Corporation), 19 May, 1998 (19.05.98), Full text (Family: none)	1-14
A	US, 5607934, A (Otsuka Pharmaceutical Co., Ltd.), 04 March, 1997 (04.03.97), Full text & JP, 7-25858, A Full text & WO, 95/2593, A1 & EP, 659182, A & AU, 9470832, A & CN, 1112364, A	1-14
X	Hiroshi Kanzaki, Daisuke Imura, Reiko Sashida, Akiko Kobayashi, Kazuyoshi Kawazu, "Effective Production of Dehydro Cyclic Dipeptide Albonoursin Exhibiting Pronuclear Fusion Inhibitory Activity" The Journal of Antibiotics (1999), Vol. 52, No. 11, pp. 1017-1022	9-11
X	Hiroshi KANZAKI et al., "Housenkin no Kouso ni yoru Kanjou Dipeptide rui kara Datsu Suiso gata Kanjou Dipeptide e no Biseibutsu Henkan"	9-11

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 November, 2000 (29.11.00)	12 December, 2000 (12.12.00)
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer
Facsimile No.	Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP00/06807

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Nippon Nougei Kagakukai Kansai Shibu Taikai oyobi Symposium Youshishuu (1999),p. 48	
X	Hiroshi KANZAKi et al., "Shinki no Kanjou Dipeptide Datsu Suiso Kouso to sono Kassei Sokutei hou no Kakuritsu" Okayama Daigaku, Nougakubu, Gakujutsu Houkoku (1999), Vol. 88, pp. 7-11	9-11

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP00/06807

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 9-11
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

See extra sheet.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP00/06807

Continuation of Box No.I-2 of continuation of first sheet (1)

Although the inventions as set forth in claims 9 to 11 pertain to dehydrogenases, it is merely stated in the specification that these enzymes may originate in any organisms such as *Streptomyces albulus*. Since enzymes have substrate specificity, the particular scope of the enzymes cannot be specified, other than those particularly cited in the description, though the common general technical knowledge on the filing day is taken into consideration. Thus, no meaningful international search can be performed thereon.

Form PCT/ISA/210 (extra sheet) (July 1992)